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FACULDADE DE CIÊNCIAS
DEPARTAMENTO DE BIOLOGIA ANIMAL

The variable behavioural and physiological outcome of social challenges in two species of Brazilian damselfish

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2017

À minha Mãezinha Paula
e à Vóvó Luísa

Agradecimentos

Ao meu orientador, Carlos Assis por estar sempre presente e não me deixar desistir nos momentos mais difíceis. Por ter sempre uma palavra de apoio e nos fazer trabalhar nos momentos em que não queríamos tanto.

À minha co-orientadora, Marta Soares e à Sónia Cardoso por me terem dado a oportunidade de embarcar nesta aventura e por me terem recebido na sua casa e apoiado em tudo o que puderam.

Ao Professor Carlos Eduardo Ferreira (Cadu), da Universidade Federal Fluminense por me receber na base de Arraial do Cabo e me proporcionar momentos de pura discussão científica mesmo às tantas da noite e por me ensinar a duvidar sempre do meu trabalho de modo a melhorar cada vez mais. Obrigada pela oportunidade de aprender mais e melhor.

Ao Professor Leonardo Barcellos, da Universidade de Passo Fundo por toda a ajuda com as análises de cortisol e ajuda na resolução de problemas e contratempos que surgiram durante todo o processo. Obrigada por não deixar que este trabalho tenha sido em vão.

Professora Maria João Santos, da Faculdade de Ciências da Universidade do Porto pela disponibilidade e interesse demonstrado no nosso trabalho e por me dar a conhecer uma pequena parte do gigantesco mundo dos parasitas em geral.

À minha querida Francesinha Pauline e ao Miguel, o Açoreano de São Miguel por serem os meus colegas de casa durante três difíceis meses de trabalho, por toda a ajuda no campo, por toda a paciência nos dias menos bons e por todo o apoio em todas as alturas. Tenho a certeza que sem vocês não teria sido tão divertido e produtivo. Obrigada por tornarem aqueles dias que estavam "maus que mete meeeedo" (ler com sotaque açoreano sff.) menos assustadores.

À minha querida amiga e confidente Renata Mazzei por ter recebido uma completa estranha no Brasil com o maior abraço e sorriso do mundo e por me deixar ser eu mesma, sempre. Obrigada pelos pequenos almoços mais maravilhosos de sempre, pelas brincadeiras, pelos passeios, pela ajuda e também pelos momentos menos bons que tenho a certeza que nos aproximaram ainda mais. Tens sempre uma palavra de apoio e conforto qualquer que seja o problema e a tua energia positiva e determinação contagiam qualquer pessoa, obrigada por seres uma inspiração para seguir os meus sonhos. Obrigada por todos os momentos que passámos juntas e por sentir que aos poucos temos criado e vamos criar uma amizade além fronteiras. Obrigada

À minha Família Brasileira, Ana Mazzei, Fernando, Bruno Ragi, Thais e todos os outros por me terem recebido na sua casa e nas suas vidas de braços abertos e me terem dado a conhecer tão bem a cultura, costumes e locais brasileiros. Deixaram muitas saudades e vontade de voltar para vos visitar e retribuir tudo o que fizeram por mim. Um obrigada genuíno do fundo do coração.

Ao "team" da Casa Base, Bia, César, Dudinha, Laís, Linda, Marcos, Matheus, Moisés, Nara, Thiago, Ricardo Sorrisos, Vini, Capitão Marcelo e tantos outros que me receberam super bem em Arraial e tornarem a minha estadia aí muito melhor. Obrigada por toda a ajuda no trabalho de campo por madrugarem por causa dos meus mergulhos, por toda a aprendizagem e por todos os momentos maravilhosos que passámos juntos sem vocês não teria sido a mesma coisa. Obrigada por tornarem estes 3 meses nos melhores do mundo!

À minha Pocinhas e à minha Tété (e Messi) por terem sido as minhas companheiras nº 1 deste "desafio/problema/feito" que me compreendem mais que ninguém e que passam por tudo comigo. Obrigada por nunca me deixarem desesperar sozinha e por me acompanharem nesta montanha russa de emoções chamada Tese em Ecologia Marinha. Estamos juntas até ao final (mas apenas em pt)!

À minha psicóloga preferida, Catarina Luz por me ter acompanhado em todas as fases daquele que sinto que foi um dos anos mais desafiantes da minha vida e por me ter ensinado sempre a ver o lado positivo de cada situação e quando tal não foi possível, chorou, berrou e mandou o mundo inteiro a um sítio feio comigo. Obrigada pelas "horas extras" que fizeste, pelas conversas de apoio e por todos os programas que fizemos para me manter minimamente sã durante todo este processo. Sabes que és muito importante e sem ti tudo teria sido mais difícil. Um obrigado nunca chegará.

Às minhas eternas amigas viajadas Andreia Margarido (maninha), Maria Lilas Matos, "Lu Bidu" Ramalho, Teresinha Teles Branco e à minha "Princesa mais linda" Joaninha Clara Silva por nunca me deixarem desanimar e fazerem com que a Europa pareça muito mais pequenina do que o que é realmente. Obrigada por terem iniciado este ciclo comigo há 7 anos atrás e por nos dias de hoje me encherem de orgulho em tudo o que fazem. Apesar de cada uma estar num local diferente e com um objectivo diferente todas estão a seguir os seus sonhos e isso é o mais importante...obrigada por serem uma inspiração!

Às minhas amigas Andreia Duarte (Intensa), Raquel Frazão (Kunanga da minha vida), Martinha (Palmeirinhas), Mimi (Oh meu Deus!), por serem a parte mais positiva de ter atrasado um ano da minha licenciatura, foi um prazer enorme poder partilhar estes anos convosco e por tornarem tudo melhor..são uma lufada de ar fresco na minha vida.

À minha turma de mestrado por nunca me deixarem sentir que estou só nesta viagem, por me acompanharem em cada etapa deste que parece ser o maior desafio até ao momento e por ter a certeza que mesmo separados fomos uma turma bastante unida e da qual sentirei imensa falta quando chegar o fim.

Ao grupinho forte de trabalho, Leminhos, Gameiro, Maria (a psicóloga), Messi (só às vezes), Pocinhas, Ritinha, Tété, Zezolas, por terem sido a minha companhia de todos os dias e desesperos e por, passado um ano consecutivo a tentar trabalhar na fcul, decidirmos ir fazer "terapia de grupo" para a faculdade de Psicologia. Sei que vamos conseguir chegar ao fim desta etapa. Obrigada por tornarem os meus dias melhores.

À minha família de Vairão, Tété (a fofinha) e Messi (o guardião da floresta), por terem sido a melhor companhia possível durante a nossa vivência na vila dos muros altos. Por todos as refeições, todos os desabafos, todos os jogos de Warframe para libertar os nervos, todos os passeios até Vila do Conde (sim, porque o Porto era looonge, fica para conhecer noutra altura, ahah), todos os fins de semana de pausa a andar de bibicleta e geocaching e acima de tudo por todo o apoio nas fases mais difíceis. Sei que muitas vezes vos levei ao "desespero" e por isso mesmo um Obrigada gigante.

À Família Alves por terem sido a família que "sempre quis e que nunca tive". Obrigada por me apoiarem nas minhas escolhas e por me terem tratado sempre tão bem. Obrigada por acreditarem em mim.

Aos meus "anjinhos" Leminhos, Mike, Pepe e Pocinhas por todos os "Irish" a horas tardias, por todas as conversas, por ouvirem todos os meus desesperos, por todos os abre-olhos necessários,

por todos os programas de fim de semana e por todos os momentos que me tiraram de casa quando mais precisava. Obrigada por me receberem de braços abertos no vosso grupinho, por nunca me deixarem sentir sozinha e mais importante que tudo por não me deixarem desistir de nada. Um simples obrigado não chega..

A todo o pessoal do Jardim Zoológico de Lisboa por ter sido um escape maravilhoso e um local de trabalho e aprendizagem durante este tempo.

A toda a equipa do Dez prá Uma por tornarem os meus dias de trabalho os melhores do Mundo! Às minhas meninas de Campo de Ourique, Inês Matos (Máátos!), Inês Branco, Sameirinho, Luisinha e Enize (a minha pasteleira preferida!) obrigada por me conseguirem por sempre a sorrir com tanto disparate, é um previlégio trabalhar convosco. Por último mas não menos importante, à minha colega e amiga de sempre Teresinha Teles Branco por me ter arranjado este espacinho tão especial a trabalhar com gente maravilhosa, Obrigada.

À Família Leonardo Melo por me ter recebido de braços abertos na sua casa e nas suas vidas, por me fazerem sentir segura e feliz sempre que estou convosco e por não nos deixarem desistir nunca desta tese interminável, Obrigada.

Ao meu Mauro (Maurinho) por ter sido um grande apoio na fase final deste desafio, por ser o meu "motivador pessoal", por ser capaz de puxar por mim todos os dias e ficar feliz com todas as pequenas vitórias, sempre com o maior sorriso do mundo na cara. Obrigada por me teres tirado de casa quando mais precisei, mesmo sabendo que devíamos trabalhar, por teres tornado este desafio uma conquista a dois e acima de tudo por me fazeres voltar a sorrir e a acreditar que tudo é possível...Obrigada.

Quero ainda agradecer à minha "Mãezinha" por me apoiar sempre em todos os momentos da minha vida, por ser um exemplo e inspiração e por ter sido desde sempre a Mãe e o Pai que sempre precisei. Obrigada por me teres incentivado a seguir os meus sonhos (mesmo que pareçam loucos) e a nunca desistir por mais difícil que seja o caminho. És uma inspiração enquanto pessoa, obrigada! Obrigada à minha Avó do fundo do coração por ter tido sempre o maior orgulho na sua neta que faz "mergulho muiiito profundo" e por gritar isso aos 4 cantos do mundo. Obrigada ainda por ter perguntado todos os dias como ia o meu trabalho apesar de até hoje não compreender o que faço a não ser "trabalhar com peixinhos".

Resumo

Todos os seres vivos poderão em algum momento no seu ciclo de vida experienciar algum tipo de *stress*, que pode ser definido como uma reacção a um estímulo desconfortável, físico ou psicológico (*stressor*), que afecta a homeostasia de um determinado indivíduo. Esses mesmos indivíduos estão constantemente expostos a esses agentes causadores de *stress* no seu habitat no meio natural, ou mesmo em condições laboratoriais, apesar de estes diferirem muito entre ambientes. Geralmente, os stressores desencadeiam uma reacção que provoca uma série de reacções bioquímicas e fisiológicas a nível do indivíduo de modo a que este recupere o seu equilíbrio homeostático. As reacções a esses estímulos variam muito entre espécies de acordo com a magnitude, tempo de exposição e origem do *stressor*. Os peixes são considerados animais mais sensíveis a possíveis alterações do meio, uma vez que estão em permanente contato directo com o mesmo devido às suas características morfológicas (corpo coberto por muco, escamas e pele). Essas características tornam-os mais susceptíveis a alterações do meio ambiente podendo influenciar os seus níveis de *stress*, reforçando assim a importância da realização de estudos que permitam compreender de que forma os níveis de *stress* são afectados por variáveis bióticas e abióticas. Existem inúmeras variáveis que podem influenciar os níveis de cortisol nos peixes e consequentemente o seu *stress*. As espécies territoriais, como as donzelas, por exemplo, podem sofrer um aumento nos níveis de cortisol devido às contínuas intrusões no seu território por parte de outros peixes, que podem expulsar mais ou menos activamente. A carga parasitária é também outro dos factores que pode induzir *stress* nos peixes uma vez que os ectoparasitas se fixam na superfície corporal do hospedeiro e alimentam-se do seu sangue e tecidos, sendo mais frequentemente encontrados nas brânquias e barbatanas. Todos os ecossistemas de recifes de coral saudáveis são compostos por uma diversa comunidade de parasitas, contudo, essa comunidade irá afetar negativamente outros seres vivos marinhos. De modo a tentarem reduzir a sua carga parasitária, os peixes infectados, denominados geralmente por clientes, podem visitar estações de limpeza. Nestas estações de limpeza, os peixes-limpadores, como por exemplo algumas espécies de góbios (*e.g. Elacatinus figaro*) alimentam-se dos ectoparasitas dos peixes-clientes que as visitam no intuito de reduzirem a sua carga parasitária. No entanto, apesar de existir uma redução da carga parasitária promovida por esses limpadores, essa redução nem sempre implica uma diminuição nos níveis de *stress* certa e óbvia. Embora as espécies territoriais que possuem uma estação de limpeza no seu território possam beneficiar de um acesso mais fácil aos limpadores, podem também sofrer um aumento nas intrusões no seu território por potenciais clientes o que torna difícil de avaliar o resultado dessas interacções. A presente dissertação teve assim como objectivo principal medir os níveis de *stress* em duas espécies de peixes-donzela territoriais endémicos do Brasil, pertencentes à família Pomacentridae (*Stegastes fuscus* e *Stegastes pictus*) e compreender se variáveis como a carga parasitária e as intrusões no seu território iriam influenciar esses mesmos níveis de *stress*. Adicionalmente, tentou-se compreender se existiria algum grupo trófico que invadisse mais os territórios destas espécies e se, por ventura, juntamente com o tamanho do intruso induziriam uma resposta mais reactiva por parte das donzelas. Tentou-se ainda determinar se variáveis como temperatura, carga parasitária, tamanho do indivíduo e área do território influenciavam o comportamento alimentar das donzelas (*foraging behaviour*). Testou-se se a presença de uma estação de limpeza dentro do território de uma donzela teria influência na frequência de intrusões por outras espécies de clientes nesse mesmo território. Por último criou-se um modelo de modo a testar quais seriam as variáveis que influenciavam os níveis de cortisol de modo a tentar identificá-las como potenciais fontes de *stress* elevado. As amostragens foram recolhidas num recife rochoso em Arraial do Cabo, Brasil durante três meses. Foram recolhidos dados

sobre variáveis abióticas relacionadas com o território, comportamento dos indivíduos, carga parasitária e níveis de cortisol. Foi ainda registada a presença de estações de limpeza no território de cada donzela observada e, sempre que houve uma procura por parte da donzela de uma estação de limpeza (dentro ou fora do seu território), registou-se também a frequência e tempo de limpeza despendido pelos góbios limpadores. No total foram observados 40 indivíduos, 20 *S. fuscus* e 20 *S. pictus*, durante 15 minutos, em que se analisaram quais as espécies de intrusos que invadiam esse território bem como o seu tamanho e a reacção da donzela à entrada desse mesmo indivíduo. Após concluídas as observações comportamentais os indivíduos eram capturados e levados para laboratório para que a carga parasitária de cada donzela fosse avaliada e identificada com o uso de uma lupa de dissecação, sempre que possível até à família. Finalmente, os níveis de cortisol de ambas as espécies foram também avaliados em laboratório recorrendo a uma análise de "*whole-body cortisol*". As diferenças entre espécies foram analisadas recorrendo a testes de Mann-Whitney, enquanto que as diferenças entre grupos tróficos foram analisadas recorrendo a testes de Kruskal-Wallis. Por último, foram ainda elaborados alguns modelos estatísticos *glm* (*general linear models*) de modo a responder a algumas perguntas mais específicas, tal como quais seriam as variáveis que influenciavam os níveis de cortisol das donzelas em estudo. Diferentes funções (Gaussiana, Poisson e Binomial) foram usadas considerando as diferentes distribuições das variáveis dependentes em estudo. Apesar da frequência de intrusões não apresentar diferenças entre as espécies de donzelas estudadas, geralmente os territórios de *S. pictus* foram invadidos por intrusos maiores. Considerando o comportamento agonístico inerente a estas espécies territoriais, *S. fuscus* foi significativamente mais reactivo quando comparado com *S. pictus*, perseguindo e expulsando mais intrusos dos seus territórios. Além disso, descobriu-se que a frequência de *chases* era negativamente afectada pelo tamanho dos intrusos que invadiam os territórios das donzelas, quanto maior os intrusos menos eram perseguidos e expulsos dos territórios. Considerando a frequência de ectoparasitas de cada indivíduo não foram encontradas diferenças significativas na carga parasitária entre as espécies de donzelas o que poderá ser explicado pela proximidade ao solo a que ambas as espécies habitam. Por último foi ainda descoberto que as estações de limpeza parecem ter uma influência positiva na frequência de intrusos nos territórios das donzelas, apesar de não ser possível generalizar estes resultados, uma vez que a amostragem precisaria de ser mais homogénea. Assim sendo, num estudo futuro seria importante ter este factor em consideração. Contudo nenhuma das variáveis em estudo revelou ter uma influência nos níveis de cortisol o que deixa bastantes portas abertas para estudos futuros. Sendo já uma temática bastante estudada em zonas como as Caraíbas e Austrália, por exemplo, este estudo constitui-se como uma das primeiras explorações em Arraial do Cabo relativa à avaliação dos níveis de *stress* em ambiente natural. Esta zona é afectada por condições hidrológicas de especial interesse (fenómenos de correntes de *upwelling*) potenciando assim as diferenças de temperatura, visibilidade e nutrientes que poderão influenciar os níveis de *stress* dos seres vivos que aqui habitam, tornando-a assim um local de interesse para o presente estudo. Os resultados obtidos através deste estudo poderão ser um ponto de partida na origem de mais estudos complementares, que irão adicionar mais informação sobre o comportamento em espécies territoriais e ajudar a compreender melhor quais as variáveis que poderão influenciar o *stress* em peixes territoriais.

Palavras-chave: *Stegastes*, territorialidade, comportamento, níveis de *stress*, ectoparasitas.

Abstract

Stress, as a reaction to an uncomfortable stimulus, promotes a set of physiological and/or behavioural responses to overcome potential threats and restore equilibrium. These responses vary among species according to magnitude, time of exposure and nature of the stressor. Territorial species, such as damselfishes, may experience an increase on cortisol levels by the continuous intrusions on their territory by other fishes, which they may chase. Ectoparasites are also capable of inducing stress by feeding on their host blood. To lower ectoparasite loads, infected fish - clients - may visit cleaning stations, however the decrease in stress levels promoted by cleaners is not always certain or obvious. While territorial species with a cleaning station on their territory may profit by having an easy access to cleaners, they may also have an increase of intrusions by other potential clients. The present study aimed to understand the outcome of these interactions and also try to learn if variables such as ectoparasite loads and/or territory intrusions influence cortisol levels in territorial species. Territories of two damselfish, *Stegastes fuscus* and *Stegastes pictus* from one rocky reef in Arraial do Cabo, Brazil, were sampled. Data about intrusions in the territory, agonistic and feeding behaviour, ectoparasite loads and territory area were collected and analysed. Furthermore, a whole-body cortisol analysis was performed. Although intrusions frequency did not present significant differences between damselfish species, *S. pictus* territories were invaded by larger intruders. Regarding agonistic behaviour *S. fuscus* was significantly more reactive when compared with *S. pictus*, chasing more intruders from their territories. Furthermore, chase frequency was found to be negatively affected by size of the intruder, the larger the intruders the less they were chased. No significant differences were found in ectoparasite loads between damselfish species. It has been found in this study that cleaning stations seem to have a significant effect in intrusions frequency in damselfish territories. However, none of these variables appeared to influence cortisol levels. These results should lead to further complementary studies, which would add more information on the behaviour of key territorial species and better understanding of the variables that may influence stress in fish.

Key words: *Stegastes*, territoriality, behaviour, stress levels, ectoparasites.

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1. INTRODUCTION

1.1. The stress response in fish

Stress can be defined as a physical, emotional or mental condition caused by the reaction to an uncomfortable physical or psychological stimulus (stressor), which can be intrinsic or extrinsic to the organism (Bonga, 1997). This physiologic response interferes with the dynamic equilibrium of the organism (known as homeostasis) and consequently there is an attempt of adaptation to the stimulus in order to restore the homeostatic equilibrium (stress) (The American Heritage ® Science Dictionary, 2017). Thus, stressors hold a dual function, not only they influence and disturb homeostatic balance of organisms, but they also promote a set of physiological and behavioural adaptive responses allowing individuals to overcome the threats and restore homeostasis (**Figure 1.1**) (Bonga, 1997).

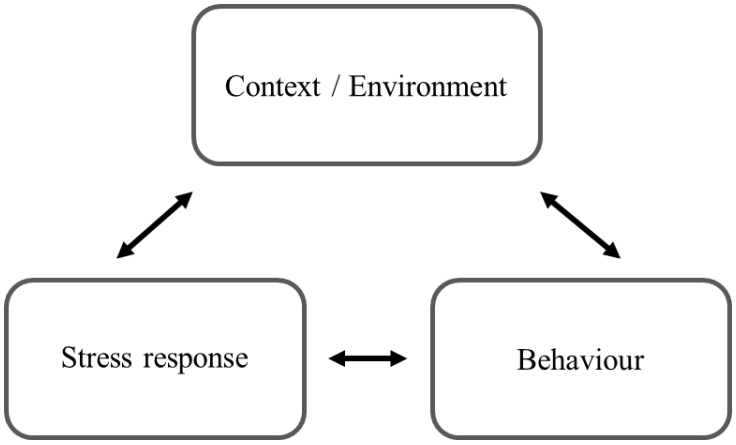

Figure 1.1 - Schematic representation of the reciprocal relationship (model) between stress and behaviour.

1.2. How are animals stressed?

Any abiotic or biotic agent may be considered as a stressor as long as it triggers a stress response by the individual (Wedemeyer *et al.*, 1990 in Moreira and Volpato, 2004). Indeed, individuals are constantly exposed to these stressors both in nature and in artificial conditions, such as laboratories and aquaculture, although they differ in each environment (Iwama, 1998). Although stress in teleost fish shows some similarities with terrestrial vertebrates (Bonga, 1997) it is known that different species react differently to the same stressor, specifically according to the type and magnitude of stressor and the period of exposure to that same stressor. Ultimately the impact of stressors, depends mainly on the behaviour of the stressed organism to the stressor and its mechanisms of recovery (Lafferty and Holt, 2003).

Stressors affecting fish were classified by Moreira and Volpato (2004) in four different types: 1) biotic or physical factors that act directly on the animal, such as bites and fights (classified as social stress), abiotic factors (temperature variation and hypoxia conditions), and handling and confinement in laboratory individuals; 2) physical factors with no direct contact with the animal, such as chemical, visual or sound cues from the stressor (*e.g.* a predator); 3) cues from stressed conspecifics imposed by any of the previous stressors; 4) Non stressful

stimulus that act as a stressor, triggering memories of a physical stressor previously known by the individual, which may not be currently present (Moreira and Volpato, 2004; Barcellos *et al.*, 2011).

1.3. Stress response in teleost fish

The stress response enables animals to handle with potentially disturbing situations that may differ in source (physical or social) (**Figure 1.1**). Generally stressors trigger an alarm reaction which invoke a series of biochemical and physiological reactions (Bonga, 1997). In addition to these biochemical and physiological reactions, stress response in fish involves all levels of organization (cell, individual organism, structure of population). Besides, it can be shown at a behavioural and/or physiological level, although behavioural changes are more debatable and species-specific than physiological parameters (Bonga, 1997; Iwama, 1998; Barreto and Volpato, 2004). At the organismal level, when exposed to stress, fish may change their behaviour as a mitigation mechanism to stress exposure (*e.g.* avoid predators) or rather activate the interrenal axis (hypothalamic-pituitary-interrenal tissue axis (HPI)), which leads to an increase of corticosteroid production. This physiological cascade is usually described as being able to elicit one of two alternative behavioural responses: a proactive response (active coping, or 'fight-flight') or a reactive response (passive coping, or 'conservation-withdrawal'), (Boer and Koolhaas, 2003).

Differences in coping behaviour are usually linked with distinct stress responses: pro-activity presumes high sympathetic reactivity and low HPI activity, whereas reactivity is associated with low sympathetic reactivity and high HPI activity. For instance, cleaning gobies respond to stressful event (facing a predator) by approaching predators faster and interacting for longer periods (Soares *et al.*, 2012).

The stress response in fish is commonly categorized in three phases (Iwama, 1998). In primary stress response, at the organism level, there is an altered state of homeostasis that commonly origin a neuroendocrine response characterized by a rapid release of hormones into circulation such as catecholamines and cortisol (Sumpter, 1997; Iwama, 1998; Mommsen *et al*. in Basu *et al.*, 2001). Primary and secondary stress responses are intimately related, the second being characterized by biochemical and physiological effects associated with stress. Stress hormones released into the bloodstream, during primary stress response, activate diverse metabolic pathways, resulting in alterations in blood chemistry and haematology, thus constituting the secondary stress response. One of the main indicators of metabolic effects due to stress is the increase of glucose concentration in the plasma, important to provide more energy to tissues (brain, gills and muscles) essential to cope with the increase energy demand (Iwama, 1998).

It is known that stress is an energy demanding process where individuals mobilize energy to handle with uncomfortable situations (Gamperl *et al.*, 1994; Schreck, 2010; Sokolova *et al.*, 2012). This higher energy consumption is essential to cope with stress itself. Cortisol may be used to mobilize this stored energy in order to sustain vital processes (Bonga, 1997). However, when stress is chronic and individuals are over the effect of stressors during indefinite time and are unable to acclimate to these same stressors, stress response loses adaptive value and becomes dysfunctional (Bonga, 1997). This leads to changes at the organism and population level, as a result of repartition and reallocation of energy away from vital processes, such as growth and reproduction (Chrousos and Gold, 1992; Barcellos *et al.*, 2007).

Changes at these levels establish the tertiary stress response, and may influence and alter the community species abundance and diversity, by decreasing recruitment and productivity, while individuals may also suffer a decrease in resistance to pathogens (Bonga, 1997; Iwama, 1998), due to implications to the immunological system (Ros *et al.*, 2010).

Fish may be more affected by stress that other animals, since they are always in direct contact with the environment, through gills, tegument (mucus and scales) being more sensitive to alterations in the environment (Schreck, 1981; Schreck, 1990 in Bonga, 1997). Fish have highly sensitive perceptual mechanisms (*e.g.* lateral line system) that detect small variations in the surrounding environment (movement, vibration, pressure and temperature gradients) and these variations may induce an alteration in individual homeostasis and therefore a stress response (Bonga, 1997). Thus, fish are appropriate models to test for stress response in relation to putative alterations of environment and behavioural conditions.

1.4. Stress hormones

There are several stress hormones that can be release during the primary stress response, such as catecholamines (adrenaline, noradrenaline, dopamine) and corticosteroids (cortisol, which is a steroid) (Mazeaud *et al.*, 1977; Gamperl *et al.*, 1994). All of them, (plus glucose) have been well stablished as physiological indicators of stress in fish (Barreto and Volpato, 2004). Still, cortisol is widely accepted as the main stress-hormone in vertebrates (Ellis *et al.*, 2004; Wong *et al.*, 2008; Nusbaumer, 2013). This stress hormone belongs to the glucocorticoid class of hormones (Linden *et al.*, 2008; Nusbaumer, 2013). Cortisol is released from the interrenal tissue (located in the head kidney) in stress conditions and/or lower blood concentration of glucose, in response to several pituitary hormones (*e.g.* ACTH – adrenocorticotrophic hormone) (Bonga, 1997; Randall *et al.*, 1997; Iwama, 1998).

As main functions, cortisol stimulates amino acids mobilization from muscles, increases transfer of fatty acids from adipose tissue to liver, and promotes gluconeogenesis in liver to raise blood's glucose concentration (Randall *et al.*, 1997). As a result, blood sugar will increase in response to this hormone secretion into the bloodstream and this increase turns into a burst of energy that prepares the individual for an emergency situation (Randall *et al.*, 1997; Iwama, 1998).

1.5. Ectoparasites and stress in fish

Organisms normally establish complex relationships, occasionally with some degree of dependency between them (*e.g.* symbiosis, commensalism, parasitism) (Roberts and Janovy Jr., 2009). These relationships vary widely between different species. Parasitism can be defined as an association between two organisms, typically from different species, where one of the organisms, the parasite, lives at the expense of the second, the host, being the host negatively affected by the parasite. Present in all kind of environments parasitism is considered a successful association, and it is believed that almost every species is parasitized by at least one other species (Hudson *et al.*, 2006; Roberts and Janovy Jr., 2009; Demopoulos and Sikkel, 2015). Despite the fact parasitism is present in all environments, its importance and role in the ecosystems continues to be very controversial among authors. Although, studies in this area have focused mainly on parasite taxonomy and host distribution

so far, it is known that a healthy coral reef ecosystem is indeed composed by a diverse parasite community (Sun *et al.*, 2012).

Ectoparasite load is one of the many factors that may influence stress levels in fish. The ectoparasites attach to the host body surface and normally feed on its blood and tissues (Sikkel *et al.*, 2009), being more frequently found on the gills and fins (mostly in pectoral and anal fins, followed by caudal, dorsal and pelvic fins), with a small percentage being attached to the head and/or body of the fish (Potts, 1973 in Soares *et al.*, 2007; Rohde, 1980 in Soares *et al.*, 2007; Smit *et al.*, 2003).

The Isopoda are a wide-spread crustacean order occurring from the intertidal zone to the continental shelf and the deep sea, being found from polar to tropical waters (Schultz, 1969 in Tanaka, 2007; Brusca & Brusca, 2003 in Tanaka, 2007). Multiple isopod species are known to be parasites of other fish species, feeding on host blood or tissue. Despite that, some isopod ectoparasites may be facultative while others are obligatory, depending completely on the hosts as energy sources (Schultz, 1969; Wägele, 1989; Brusca &Wilson, 1991; Brandt & Poore, 2003 in Tanaka, 2007).

The Gnathiidae family has been reported to parasitize a large variety of fishes from marine and fresh-water environments, and are one of the most abundant and specialized crustacean ectoparasite group present in reef environments (Grutter, 1999; Arnal and Côté, 2000; Arnal and Morand, 2001; Bshary and Grutter, 2002; Tanaka, 2007). As opposed to other isopod families, gnathiids present a biphasic life cycle (**Figure 1.2**) with a fish-parasitic larval phase (pranizae) and a "non-feeding" adult phase, living in benthic habitats. After hatching, larvae emerge from the benthos, search for a potential host and begin to feed on its blood and body fluids. When they are fully satisfied and swollen (praniza) they release themselves from the host and return to the benthos, where they do the first moult. This process repeats two more times and in the third moult the larvae suffer a metamorphosis and turn into the adult form (male or female). Gnathiid larvae and adults are morphologically very different and adults show evident sexual dimorphism (Smit, 1904 in Tanaka, 2007; Monod, 1926 in Tanaka, 2007; Schultz, 1969 in Tanaka, 2007). After metamorphosis, reproduction occurs and females lay the eggs (Tanaka, 2007).

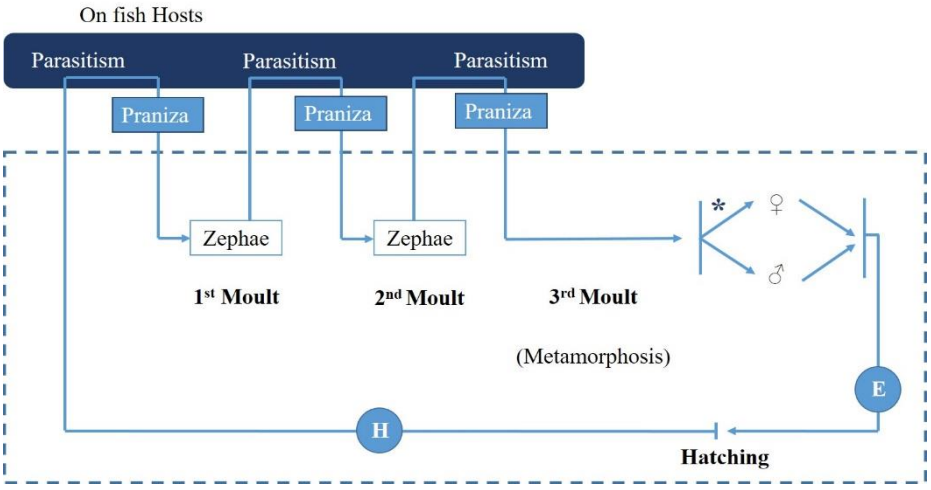

Figure 1.2 - Life cycle of gnathiid isopods (adapted from: (Tanaka, 2007)). Praniza - engorged larvae after feeding on host blood and tissues; Zephae - segmented larvae after moult and before feeding. (*) - represents the metamorphosis into an adult form; E - represents reproduction and lay of the eggs by the females; H - hatching of the eggs.

Ectoparasites, specifically gnathiids, may affect individuals in several ways, by mechanical action (wounds in tegument), withdrawal or supply of substances (anaemia), transfer of microorganisms (*e.g.* blood parasites), possibly toxic effects, and effects on the host immune response, such as increasing susceptibility to disease and decreasing in host vital rates (*e.g.* reproduction) (Rohde, 1984; Lafferty and Holt, 2003). These effects induce an increase in stress levels of individuals (Noga, 2000; Conte, 2004). Plus, since gnathiids are considered generalist feeders with preference for some species they present a serious risk to marine fishes in general (Overstreet, 1981).

Moreover, gnathiids are a key participant in cleaning symbioses as they are the major and sometimes preferred food item (Soares *et al.*, 2010) of cleaners (Cheney and Côté, 2003a; Becker and Grutter, 2004; Clague *et al.*, 2011; Waldie *et al.*, 2011; Losey, 2015) and are referred to influence the interaction between host and cleaners (Grutter, 1999; Sikkel *et al.*, 2004, 2005).

1.6. The influence of cleaning behaviour to host fish stress

Another example of complexity is the mutualistic relationships between organisms, namely the cleaning interactions in marine environments. Cleaning behaviour are described as interactions between a smaller fish or shrimp, known as "cleaner", which removes ectoparasites, injured tissue, bacteria and other particles of cooperating and usually larger organisms, known as "clients" (Vaughan *et al.*, 2016). These interactions usually take place at specific sites called cleaning stations, where cleaners may be found by their visiting clients.

There are different cleaner species, some are obligatory – that is, clean their entire live (*e.g. Elacatinus figaro*), or facultative – clean during their juvenile stage (*e.g. Pomacanthus paru*) (Sazima *et al.*, 1999, 2000; Sampaio *et al.*, 2017). Despite these differences, all of them seem to derive benefit from these cleaning interactions since they feed on ectoparasites. On the other hand, clients may also benefit from these interactions via reduction in ectoparasite load, which in turn may lead to a higher body condition and a decrease in their stress levels (Sun *et al.*, 2012; Triki *et al.*, 2016). Thus, cleaning interactions play an important role in reef ecosystems since they may reduce and maintain the balance of individuals' ectoparasite load in the environment, and subsequent physiological conditions.

1.7. Territorial species and stress: the challenges they face

While theoretically, both parts (cleaners and clients) seem to be gaining benefits, in reality these benefits should change from species to species. One thing is to visit a cleaner and leave and another is to have a cleaning station within its own territory. Territorial species face a dual challenge: on one hand, having a cleaning stations within its own territory reduces the costs of searching for a cleaning station (therefore a possible decrease in ectoparasite load and stress levels), on the other hand, all other clients will need to enter their territory as well (**Figure 1.3**) (Cheney and Côté, 2001; Bshary *et al.*, 2007). For instance, the longfin damselfish visited cleaning stations less as the distance between its territory and cleaning station increased (Cheney and Côté, 2001, 2005). On the other hand, there should be a great effort to defend the territory and its resources, especially when the presence of a cleaning station increases the rate

of intrusions. Indeed, the outcome of defending a territory is yet not fully accounted, since not always the benefits of having a territory exceed the costs territorial species may face (Clifton, 1990; Santangelo *et al.*, 1997; Vullioud *et al.*, 2013). Many species defend a territory in order to benefit from an easier access to food resources, to refuge and to better reproduction conditions. Despite the evident benefits individuals may undergo several challenges. Territorial species actively have to defend their territory from intruders such as competitors or predators. Relative to the predators, fish may also use their territory as a refuge instead of chase these potential predators. Thus as the turnout of these confrontations, individuals should have an expense of energy and increase of stress levels (Barcellos *et al.*, 2007). Additionally, herbivorous territorial species have to allocate some of their time and energy maintaining their feeding patch (*e.g.* damselfishes) (Ferreira *et al.*, 1998a). Adults face another challenge during the breeding season since they may have to leave their territory in search for a suitable mate (Cheney and Côté, 2001, 2003b).

Additionally, territorial species are confined to a specific area and more close to the substratum, which may increase their susceptibility to benthic parasites (such as gnathiids) (Sikkel *et al.*, 2000).

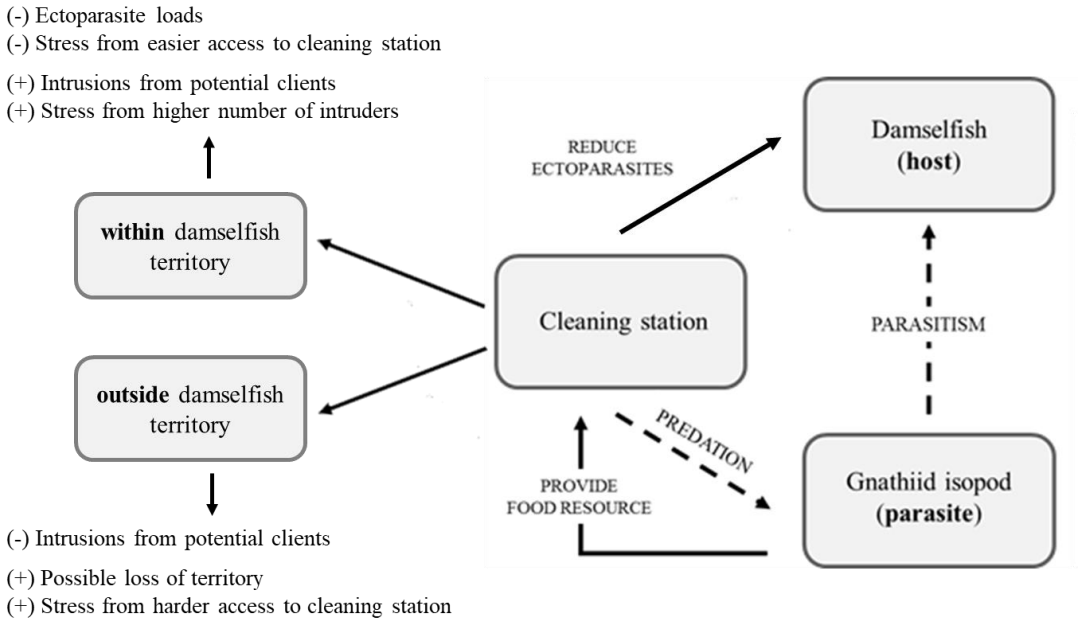

Figure 1.3 - Summary of cleaning interactions between damselfish species, cleaners and ectoparasites regarding the presence of a cleaning station in or outside the damselfish territory.

1.8. Studied species

The Pomacentridae are very diverse, including more than 300 different species, which are grouped in 21 genera. Generally they are more abundant and spatially more widespread than other conspicuous species (Robertson and Lassig, 1980; Meekan *et al.*, 1995). Fish belonging to this family are commonly known as damselfish (**Figure 1.4**) (Frederich *et al.*, 2006; Nelson *et al.*, 2016). Usually damselfish have a small mouth and one single nostril on each side, plus as a distinctive feature damselfish have a single continuous dorsal fin, usually with 11-18 soft rays following the spiny rays (Nelson et al., 2016). Damselfishes are represented by many

different species with different colour patterns, which vary with individuals and between localities within the species (Nelson et al., 2016).

Damselfish are widely distributed and, unlike other perciform families, establish an intimate relation with coral reefs, being an abundant component in these environments (Molina and Galetti, 2004; Ceccarelli, 2007). Despite this relation, they can also be found in rocky reefs, temperate regions and tropical rocky shores, all over the world (Ferreira *et al.*, 1998).

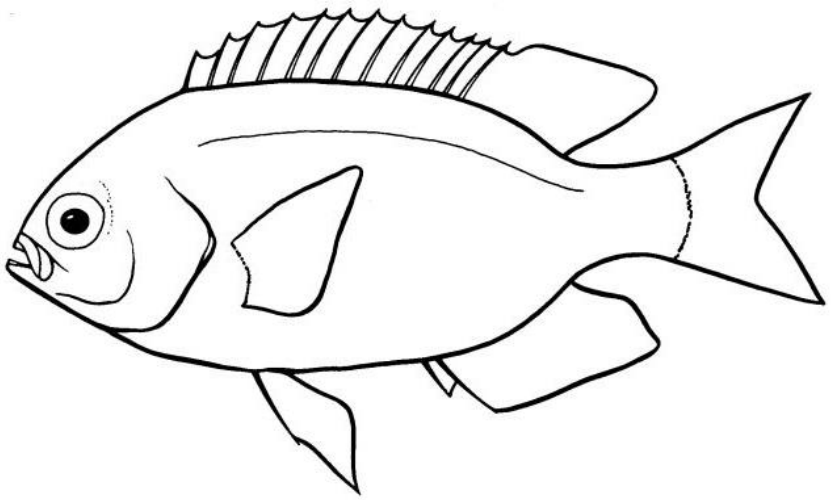

Figure 1.4 - Damselfish, adapted from (Nelson, 2006).

Damselfishes are particularly known for having a strong territorial and aggressive behaviour towards other fish that come near or cross the boundaries of their territories. Territories play an important role for damselfish, especially in terms of food resources, but they are also important in terms of refuge and reproduction conditions for these individuals (Nelson *et al.*, 2016). That is probably the reason why damselfish so actively defend their territories from all potential intruders and competitors (usually conspecifics, other herbivorous and egg predators), which may threaten their resources or their offspring (Hamilton and Lawrence, 2003; Helfman *et al.*, 2009; Souza *et al.*, 2011). With such a high diversity, some damselfish species are considered as herbivores (Floeter *et al.*, 2006; Chaves and Monteiro-Neto, 2009; Daros *et al.*, 2012), others as omnivores (Ferreira *et al.*, 2004), and still others even as invertebrate feeders (Pinheiro *et al.*, 2011; Longo *et al.*, 2014). However, this is a problem even within the same species. For instance, in the case of *S. pictus*, Ferreira *et al.* (2004), considered it as an omnivore and later in 2006 it was considered as a territorial herbivore (Floeter *et al.*, 2006).

Herbivorous fish are considered a key element in reef ecosystem ecology, and even though there is no consensus among authors regarding feeding habits of the damselfish group, their influence in the environment is undeniable. Indeed, they may be considered as "farmers" for their capacity for occupying larger proportions of the substratum and promote the selective growth of algal turf inside their territories, influencing the biomass, the productivity and even the algal composition (Ferreira *et al.*, 1998b; Ceccarelli, 2004; Ceccarelli *et al.*, 2005). Algae composition within damselfish territories can range from monocultures (Hata and Kato, 2002) to high levels of algae turfs (Hixon and Brostoff, 1983), indirectly affecting corals and other

organisms (Wellington, 1982; Zeller, 1988; Jones *et al.*, 2006). Individuals manage these "algae farms" excluding other herbivores from their territories (*e.g.* foragers, typically from families Scaridae and Acanthuridae), and selectively eating some algae species, thus controlling algae growth and composition (Ferreira *et al.*, 1998b; Ceccarelli, 2004).

According to Rocha *et al.* (2000), and Rocha and Rosa, (2001), the Brazilian Damselfish, *Stegastes fuscus* (Cuvier, 1830), and the Yellowtip Damselfish, *Stegastes pictus* (Castelnau, 1855), are two territorial species endemic to Brazil. However, some authors already reported the occasional presence of *S. pictus* in the southeastern Caribbean (Humann and Deloach, 2002). *S. fuscus* is the most abundant species in Cabo Frio Island (Ferreira *et al.*, 1998b) and can be found in shallow areas (0.3 - 15.2 m). On the contrary, *S. pictus* is found in deeper waters (6.1 - 60.9 m). Both species have similar morphology, with a "perchlike" profile and a laterally compressed body (**Figure 1.4**), although usually *S. fuscus* individuals are, slightly larger than *S. pictus* (*S. fuscus*: 7.62-12.70 cm, while *S. pictus*: 5.08-10.16 cm) (Humann and Deloach, 2002; Nelson *et al.*, 2016). Juvenile *S. fuscus* and *S. pictus* differ dramatically from adults, both in colour and in markings, the juveniles being much more colourful (Humann and Deloach, 2002). Adults from both species have a dark grey brown body colour and can be distinguished by the yellow tip on the upper half of the tail, only present in *S. pictus* (Humann and Deloach, 2002).

1.9. Objectives

The present study aimed at measuring and comparing stress levels in two territorial Pomacentridae fish species (*Stegastes fuscus* and *Stegastes pictus*) regarding: 1) biotic and abiotic variables related to their territory; 2) behavioural variables; 3) ectoparasite loads; and 4) frequency of intrusions and chases.

Additionally, it was also determined if variables such as temperature, ectoparasite load, individual's size and territory area influence foraging behaviour (used as a measure of damselfish activity).

Moreover, it was important to test if: the presence of cleaning stations within damselfish territories had an influence in the frequency of intrusions by other client species; if variables, such as intruder's size and trophic group, had an influence in damselfish reaction to intrusions; and identify which trophic group was the most predominant in intruding each damselfish species territory and which induced a more responsive reaction by the territory holder.

2. METHODS

2.1. Fieldwork

2.1.1. Study sites and species

The present study was conducted from November to December 2014 on a subtropical rocky reef at Abobrinha Bay, in Cabo Frio Island, Arraial do Cabo (22°58'S; 42°00'W), Rio de Janeiro - Brazil (**Figure 2.1**). Abobrinha Bay is included in a Natural Reserve (*Reserva Extrativista de Arraial do Cabo*) (Barbosa, 2013), a sustainable use marine reserve. This site has suitable conditions for observation studies since it is located in the west side of Cabo Frio Island, thus it is less exposed to adverse weather conditions and it is less influenced by local upwelling events (Valentin, 1984; Valentin *et al.*, 1985; Ferreira *et al.*, 1998).

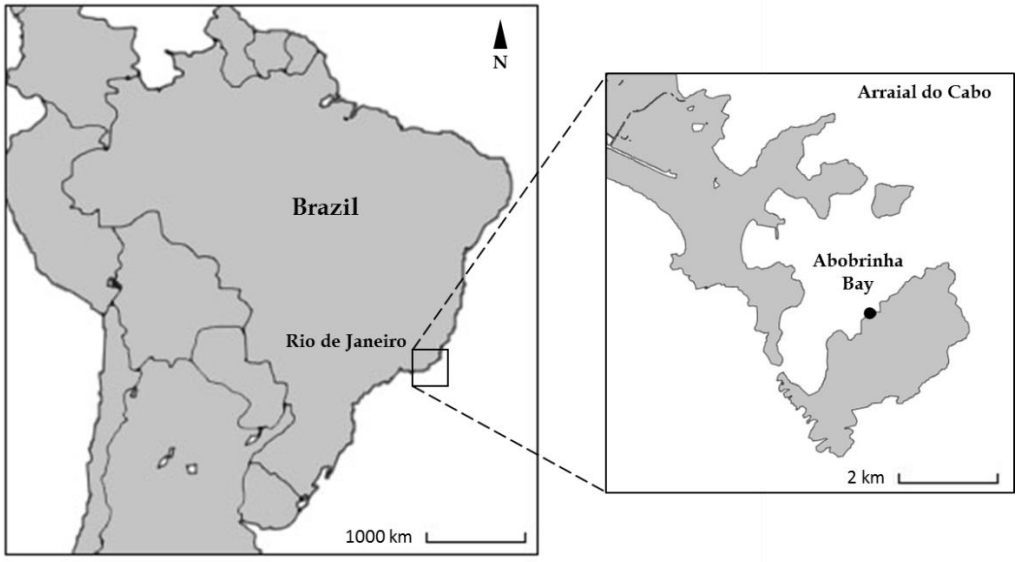

Figure 2.1 - Location of the study site. Abobrinha Bay in Cabo Frio Island, Arraial do Cabo (22°58'S; 42°00'W), RJ - Brazil.

Among the damselfish species that occur in Arraial do Cabo, *Stegastes fuscus* (Brazilian Damselfish) and *Stegastes pictus* (Yellowtip Damselfish) were chosen for this study, as they are the most abundant (Ferreira *et al.*, 2001) (**Figure 2.2**). Both species are endemic to Brazil (Rocha *et al.*, 2000; Rocha and Rosa, 2001) and easy to identify. Damselfishes are known for having a strong territorial and aggressive behaviour in keeping their territories in order to have access to food, refuge and better conditions to reproduce (Souza *et al.*, 2011). Because of the high density of these species in the studied area, the capture and sacrifice of some individuals was deemed to have minimum effects on the population level. Additionally, their easy identification as a species and their territorial behaviour make *S. fuscus* and *S. pictus* the most suitable species to achieve the aims of this study.

(a) **(b)**

Figure 2.2 - Studied species **(a)** *Stegastes fuscus* (photo credits: Carlos E. L. Ferreira) and **(b)** *Stegastes pictus* (photo credits: Marta S. Reis)

2.1.2. Behaviour observations and territory size

A total of 40 underwater behaviour observations were performed, on twenty *S. fuscus* individuals and twenty *S. pictus* individuals. Each fish was randomly selected by a roving diver and observed for a period of fifteen minutes, totaling ten hours of observation. All behavioural observations occurred between 08:00 hours and 10:30 hours, since according to Arnal and colleagues (2001) that was the most active period for the cleaners *Elacatinus* spp.. Observations took place in shallow areas (3-7 m, which was the maximum depth in this site) and began after a three minute period delay to allow the focal individual to become used to the presence of the diver (Soares *et al.*, 2012, 2007). Damselfishes were observed from a distance of at least 2 m (Cardoso *et al.*, 2009). At each observation, environmental data, such as water temperature, depth and visibility conditions (estimated visually in meters), and behavioural data were collected. Behavioural data included: a) territorial intrusions - number of intrusions, species and size of intruder (estimated visually to the nearest centimeter), b) focal damselfish response to intrusion (*e.g.* chases), c) frequency of focal damselfish bites taken on the substratum (used as a measure of damselfish activity); and d) cleaning interactions (presence of cleaning station within the focal damselfish territory, number and size of cleaning gobies (*Elacatinus figaro* Sazima, Moura and Rosa, 1997) and frequency and duration of cleaner-client interactions). Intrusion was defined as any instance in which other fish would cross damselfish territory boundaries, irrespectively of damselfish reaction. Information regarding each damselfish's territory size was also collected.

Each focal individual' territory was identified with a numbered buoy. Since territory boundaries were often obvious (Cheney and Côté, 2001), they were marked with small floats and measured in the end of each observation. To do so, two perpendicular axes (A and B) were used: each axis was drawn using the two furthest points where the individual swam to, and then the distance between the points in the same axis was measured (adapted from Aued, 2012) (**Figure 2.3**). For this, each fish's movements were visually tracked and the longest distance they travelled from their refuge along A and B axis was measured. These measurements were then used to estimate territory area using the formula for the area of an ellipse (**2.1**).

$$(Area = \pi \times semi\ major\ axis \times semi\ minor\ axis) \qquad \textbf{(2.1)}$$

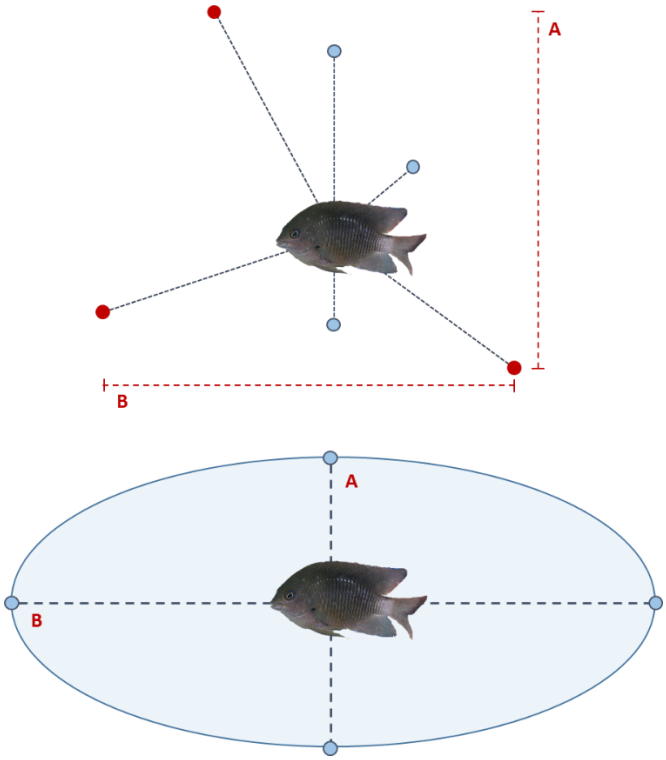

Figure 2.3 - Method of how to calculate the area of a damselfish territory.

2.1.3. Capture

After the behavioural observations and all territory measurements were completed, the individuals were collected using hand spears. This technique was considered to be potentially less stressing to individuals than other techniques (*e.g.* nets and/or hand nets) as it kept the period between capture and death of individuals as short as possible. After capture, each individual was quickly placed into a hermetically sealed plastic bag to reduce loss of ectoparasites due to handling and/or natural detachment due to host death (Grutter and Poulin, 1998; Eckes *et al.*, 2015). Each bag contained as little sea-water as possible to avoid the presence of non-parasitic organisms, such as free-living copepods. All bags were then placed in a styrofoam box with ice during transportation.

2.2. Laboratory work

2.2.1. Ectoparasite load assessment

Individuals were carried to the temporary laboratory (stablished at the boat) and, as soon as possible, the total length (T_L) of each individual was measured, ectoparasite loads were accessed and guts were removed from all individuals. In order to determine damselfish ectoparasite load, each individual and all the contents of the plastic bag were placed in a plastic container and rinsed with freshwater to promote ectoparasite detachment (Grutter, 1998; Sikkel *et al.*, 2004, 2006). The bags were then carefully rinsed with freshwater to ensure that there were no ectoparasites left, and the remaining liquids were conserved for later filtration. Next, the individuals were brushed in the opposite direction to scale intersection for several minutes (± 2 - 3 min) to remove any remaining attached ectoparasites. Gills and fins of each specimen were carefully examined to assure there were no ectoparasites attached, since these are the major

areas of settlement along with the buccal cavity, nostrils and eyes (Tucker *et al.*, 2002; Marino *et al.*, 2004; Tanaka, 2007). All liquids were then filtered with a 100 µm mesh sieve and ectoparasites were preserved in flasks (70 mL) with alcohol 70% for later identification (Sikkel *et al.*, 2000; Soares *et al.*, 2008). Samples were placed on a petri dish under a binocular microscope (40×) and all parasites were counted and identified to family level (Rohde, 2005; Soares *et al.*, 2007). In the end, all individuals whole body were preserved in a freezer (-20˚C) until cortisol analyses were performed.

2.2.2. Cortisol analysis

Cortisol levels were used as a measure of stress response, a whole-body cortisol analysis was performed using a method described by Sink *et al*., (2007). All individuals were weighed and, following Barcellos *et al.*, 2007, each one was minced and placed into a stomacher bag with 2 mL of PBS (Phosphate Buffered Saline, pH 7.4) for 6 min. Then, all the contents of the stomacher bag were transferred into a 10 mL test tube with 5 mL of laboratory grade ethyl ether. The tube was vortexed for 1 min and centrifuged for 10 min at 3000 rpm (Barcellos *et al.*, 2007; Piato *et al.*, 2011). The tube was immediately frozen in liquid nitrogen and the unfrozen portion (ethyl ether with cortisol) was decanted and stored in a new container. To extract the portion containing cortisol, the ethyl ether was completely evaporated under a gentle stream of nitrogen for 2 h. The final lipid extract containing the cortisol was then stored at -20˚C until an ELISA was conducted on the samples suspended with 1 mL of PBS buffer. Whole-body cortisol was measured in duplicate samples of tissue extract with a preexisting kit EIAgen™ CORTISOL test (BioChem ImmunoSystems). The specificity of the test was evaluated by comparing the parallelism between the standard curve and serial dilutions in PBS (pH 7.4) of the tissue extracts. The standard curve constructed with the human standards ran parallel to that obtained using serial dilutions of damselfish tissue extract (Barcellos *et al.*, 2007).

2.3. Statistical Analysis

All data treatment and preparation was performed with *Microsoft Excel* 2013. Statistical analyses were done using *IBM SPSS Statistics 22* and *R version 3.2.4 revised* (R Core Team, 2016).

For each damselfish species, there were a total of sixteen previously indicated variables collected, and two more (intruder's trophic group and proportion of chases), that were defined before data analysis.

The assumptions of normality and homoscedasticity were tested for all behavioural variables under study before preforming any type of analysis. Because none of the variables followed a normal distribution, non-parametric tests were used for all data analyses, except in the case of one specific general linear models (*glm*). The significance level was set at p-value ≤ 0.05.

Mann-Whitney tests were used for comparisons between the two damselfish species in some of the studied variables when necessary.

Kruskal-Wallis tests were performed for the two damselfish species in study as to:

1) Discover if there was a predominant trophic group that entered the damselfish territory, followed by the post-hoc Dunn test whenever necessary;
2) Understand if there were trophic groups preferentially chased by damselfish's species, followed by the post-hoc Dunn test whenever necessary.

In order to answer some of the proposed objectives in this work, several statistical models were executed. Statistical models were performed using the *lmer* function from Lme4 library and *glm* function from STATS library. Independent and dependent variables were established and correlated variables were eliminated from the model, since they add error and increase interpretation bias (Boldina and Beninger, 2016). Correlation between variables was visually analysed with the function *pairs* from graphics library, and some correlations were confirmed with the function *cor* from STATS library. Different functions (Gaussian, Poisson and Binomial) were used according to dependent variables distribution. Model validity was assured by visual analysis of residuals error distribution after running the models.

1) To determine which variables influence foraging behaviour, specifically bites on the substratum, a predictive *glm* (General linear model) was executed using a Poisson function. The model used bites on the substratum as a dependent variable, damselfish species as fixed factor and temperature, ectoparasite load, individual's size and territory area as covariates (**2.2**).

$$glm \text{ formula} = (\text{bites} \sim \text{temp} + \text{ecto\_load} + \text{dalmsel\_size} + \text{terr} + \text{damsel\_specie}) \quad \textbf{(2.2)}$$

2) To determine if intruder's size and trophic group could are correlated to damselfish response towards intruders (chases), a binomial model was chosen since the dependent variable, chases, presents a binomial response (the individual either reacted or not to the intruder). The model used frequency of chases as a dependent variable, damselfish species and intruder's trophic group as fixed factors, and intruder's size as a covariate (**2.3**). The trophic group of each intruder that entered in damselfish territories was determined according to Ferreira *et al.*, 2004

$$glm \text{ formula} = (\text{chases} \sim \text{intruders\_size} + \text{specie} + \text{trophic\_group}) \quad \textbf{(2.3)}$$

3) In order to find out if the presence of cleaning stations (CS), within damselfish territories, influenced the frequency of intrusions in the territories of both species a *glz* was performed using a Poisson distribution. Frequency of intrusions was included as a dependent variable and damselfish species and cleaning station (presence or absence) as independent fixed factors (**2.4**).

$$glm \text{ formula} = (\text{intrusions} \sim \text{species} + \text{CS}) \quad \textbf{(2.4)}$$

4) Finally, in order to investigate which variables were correlated to cortisol levels, a predictive *glm* (General linear model) was executed with a Gaussian function. The variable "cortisol" had to be log transformed and, after residual errors present a normal distribution, a Gaussian distribution was used. The model used cortisol levels (ng/g) as a dependent variable, damselfish species and cleaning stations (2 levels: presence or absence) as fixed factors and gnathiid frequency, chase proportion, intruder's size, bites, and water temperature as covariates (**2.6**). Proportion of chases", was calculated using equation (**2.5**)

$$proportion\ of\ chases = \frac{chase\ frequency}{intrusion\ frequency} \quad \textbf{(2.5)}$$

glm formula = (log (cortisol) ~ gnathiids + chase_proportion + intruders_size + bites + species + temp + CS) **(2.6)**

3. RESULTS

3.1. Damselfish biology and territory characteristics

Both studied species (*S. fuscus* and *S. pictus*) are commonly present in Abobrinha Bay and, while living at different depths, their occurrence overlaps in the depth range 3.8-5.2 m. *S. fuscus* was found 3.1-5.2 m deep and *S. pictus* 3.8-6.6 m. *S. fuscus* captured measured on average 14.3 cm (13.4-15.3 cm) and *S. pictus* measured on average 9.4 cm (8.4-10.5 cm). Observations for *S. fuscus* occurred with apparent, better visibility conditions (mean = 8 m), varying between (6-10 m) than those for *S. pictus* (mean = 6 m), and varied between (2-7 m).

Temperature varied 5 degrees across all observations (18-23˚C), with *S. fuscus* with a median temperature of 21.5˚C and *S. pictu*s with 21.0˚C.

Territorial areas varied between 0.59 m^2 and 15.83 m^2 for *S. fuscus* (mean = 5.20 m^2) and between 0.31 m^2 and 11.74 m^2 for *S. pictus* (mean = 4.43 m^2). There were no significant differences in territory size between damselfish species (Mann-Whitney, $U_{38} = 165.00$, $p = 0{,}355$).

3.2. Foraging behaviour

Overall, there was intraspecific heterogeneity in the frequency of bites on the substratum and differences between species were significant: *S. fuscus* gave on average 1.2 bites min^{-1} on the substrate while *S. pictus* gave 0.3 bites min^{-1} (Mann-Whitney, $U_{38} = 31.000$, $p < 0.001$) (**Figure 3.1**).

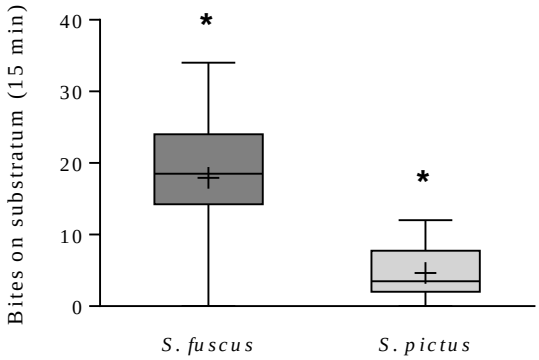

Figure 3.1 - Bites on the substratum (per 15 min of observation) of two damselfish species *S. fuscus* and *S. pictus*. Box-plots represent minimum and maximum, median and 1st and 3rd quartiles and mean is marked as "+". Significant differences are indicated with " * ". Sample size for both species is 20 individuals.

Damselfish individual's size had a positive significant effect on the amount of bites on the substratum (**Table 3.1**). Larger damselfish bit the substratum, for feeding purposes, significantly more (0.210) than smaller individuals (z = 2.210, $p = 0.027$).

Table 3.1- Summary table for *glm* using Poisson distribution relatedly to frequency of bites on the substratum (15 min). Significant values ($p < 0.05$) are highlighted in bold.

	Estimate	Standard Error	z - value	Pr(>|z|)
(Intercept)	-0.376	1,844	-0.204	0.838
Temperature	0.012	0.046	0.252	0.801
Ectoparasite load	0.003	0.017	0.153	0.879
Individual's size	0.210	0.095	2.210	**0.027***
Territory size	0.002	0.016	0.118	0.906
Damselfish Species	-0.333	0.472	-0.704	0.481

3.3. Agonistic behaviour

3.3.1. Territory intrusions

S. fuscus and *S. pictus* suffered on average 4 territorial intrusions in a 15 min observation. From all observed individuals, 90% of *S. fuscus* suffered at least one intrusion within their territory, while 95% of *S. pictus* individuals suffered at least one intrusion. Overall, no significant differences in the frequency of territory intrusions were found between damselfish species (Mann-Whitney, $U_{38} = 180.500$, $p = 0.602$).

A total, of 33 different fish species were observed entering damselfish territories. In the territories of *S. fuscus*, *Acanthurus bahianus* was the predominant species (16.88%) invading the territories, followed by conspecific individuals (15.58%). In case of *S. pictus* territories, *Pseudupeneus maculatus* (16.43%) and *Acanthurus bahianus* (13.70%) were the predominant intruders. The relative size of these intruders also varied significantly between damselfish species, with the larger intruders being associated with *S. pictus* territories. (Mann-Whitney, $U_{148} = 3426.50$, $p = 0.020$). In *S. fuscus* territories, intruders measured on average 17.0 cm in length (8.5-25.0 cm), while in *S. pictus* territories intruders measured on average 24.3 cm (3.0-100.0 cm).

There were also significant differences in the trophic groups of the intruders entering damselfish territories. Although in *S. fuscus* territories, significant differences were found between intruder's trophic group (Kruskal-Wallis test, $H_6 = 17.231$, $p = 0.008$), differences were only verified between one pair: most of the intruders were mobile-invertebrate feeders (26.67%), while planktivores was the least frequent trophic group (1.33%) (Dunn's test = 0.012) (**Figure 3.2 (a)**).

In *S. pictus* territories, most of the intruders were roving herbivores (35.62%), unlike carnivores which was the least frequent trophic group (1.37%) (Kruskal Wallis, $H_6 = 38.825$, $p < 0.001$) (**Figure 3.2 (b)**). Significant differences were also found between roving herbivores and carnivores, sessile-invertebrate feeders and planktivores; mobile-invertebrate feeders with carnivores, sessile-invertebrate feeders, planktivores and territorial herbivores (Dunn's test <0.05).

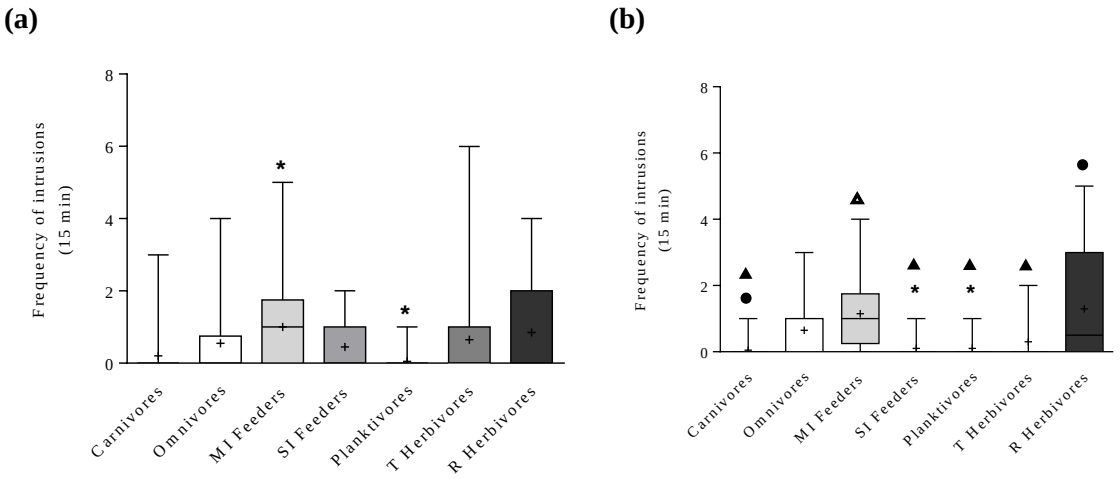

Figure 3.2 - Intrusions in **(a)** *S. fuscus* and **(b)** *S. pictus* territories by different trophic groups: Carnivores, Omnivores, Mobile-Invertebrate feeders, Sessile-Invertebrate feeders, Planktivores, Territorial Herbivores and Roving Herbivores. Box-plot represent median, minimum and maximum and 1st and 3rd quartiles. Mean is represented with "+". In *S.fuscus* **(a)** significant differences between trophic groups are indicated with " * ". In *S. pictus* **(b)** significant differences between trophic groups are indicated with "●", " ✱ " and with "△ - ▲" in which Mobile invertebrate feeders (△) differ from the other trophic groups (▲). Sample size for both species is 20 individuals.

3.3.2. Damselfish Agonistic response levels (chases)

S. fuscus chased on average more (mean = 2; min-max = 0-8) intruders than *S. pictus* (mean = 1; min-max = 0-3) (**Table 3.2**). Additionally, 10% of *S. fuscus* did not respond to the presence of intruders, which contrasts with 50% of *S. pictus* which did not chase any of the intruders (**Figure 3.3 a)** and **b)**).

Both *S. fuscus* and *S. pictus* responded more to some species of intruders than others. *S. fuscus* chased all individuals from 7 different species (*Acanthurus bahianus, Acanthurus chirurgus, Anisotremus virginicus, Halichoeres poeyi, Priacanthus cruentatus, Sparisoma axillare* and *Stegastes pictus*), on the contrary, all the individuals from another 7 species were never chased (**Table 3.2**). In data collected, *S. pictus* chased all individuals from *Chaetodon sedentarius, Hallichoeres sazimai* and *Parablennius spp*. On the contrary none of the individuals from other 16 different species were chased (**Table 3.2**).

Table 3.2 - *S. fuscus* and *S. pictus* proportion of chases related to intruder species. Trophic categories (as defined by Ferreira *et al.*, 2004 in Floeter *et al.*, 2006)

Trophic Group	Intruder species	Chases proportion	
		S. fuscus	*S. pictus*
Carnivores	*Priacanthus arenatus*	0.00	-
	Gymnothorax moringa	-	0.00
Omnivores	*Abudefduf saxatillis*	0.67	-
	Acanthostracion quadricornis	-	0.00
	Cantherhines pullus	-	0.00
	Canthigaster figueredoi	-	0.00
	Diplodus argenteus	0.00	0.00
	Parablennius spp	-	1.00
	Pomacanthus paru	0.67	-
	Sphoeroides spengleri	-	0.00
	Stephanolepis hispidus	-	0.33
Mobile Invertebrate Feeders	*Anisotremus virginicus*	1.00	0.00
	Bodianus pulchellus	0.00	0.00
	Bodianus rufus	0.00	-
	Haemolun aurolineatum	-	0.00
	Haemulon plumeri	0.33	-
	Halichoeres poeyi	1.00	-
	Hallichoeres sazimai	-	1.00
	Holocenthrus adscencionis	-	0.00
	Priacanthus cruentatus	1.00	-
	Pseudupeneus maculatus	0.33	0.08
Sessil Invertebrate Feeders	*Chaetodon sedentarius*	0.75	1.00
	Chaetodon striatus	0.60	0.00
Planktivores	*Chromis multilineata*	0.00	0.00
Territorial Herbivores	*Stegastes fuscus*	0.33	0.00
	Stegastes pictus	1.00	0.67
Roving Herbivores	*Acanthurus bahianus*	1.00	0.00
	Acanthurus chirurgus	1.00	0.50
	Acanthurus coeruleus	0.00	-
	Cryptotomus roseus	0.00	0.50
	Sparisoma axillare	1.00	0.00
	Sparisoma frondosum	-	0.00
	Sparisoma tuiupiranga	-	0.33

The level of response to intrusion varied between the two species of damselfish with *S. fuscus* chasing intruders more often than *S. pictus* (Mann-Whitney, $U_{38} = 89.000$, $p = 0.002$).

Regarding the trophic group of these intruders, no significant differences were seen in terms of chase frequencies within the same damselfish species (Kruskal-Wallis, *S. fuscus:* $H_6 = 11.719$, $p = 0.069$; *S. pictus:* $H_6 = 5.753$, $p = 0.451$) (**Figure 3.3 (a)** and **(b)**).

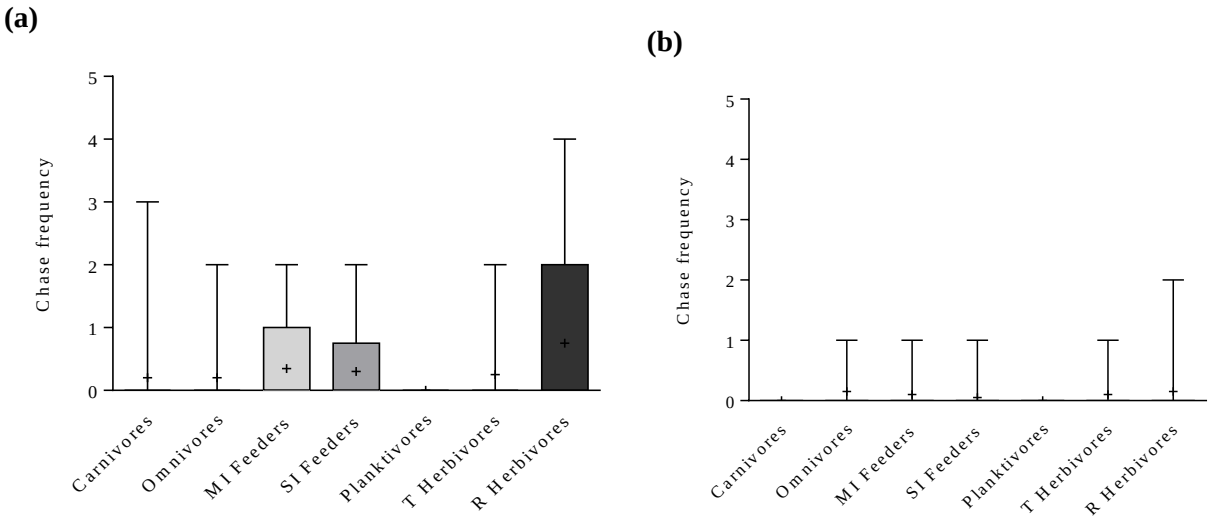

Figure 3.3 - Chase frequency in **(a)** *S.fuscus* and **(b)** *S. pictus* by different trophic groups: Carnivores, Omnivores, Mobile-Invertebrate Feeders, Sessile-Invertebrate Feeders, Planktivores, Territorial Herbivores and Roving Herbivores. Box-plot represent median, minimum and maximum and 1st and 3rd quartiles. Mean is represented with "+". Sample size for both species is 20 individuals

3.3.3. Influence of intruders size and trophic group in agonistic reactions

"Size of intruder" and "damselfish species", had a significant negative effect in damselfish reaction to the intruders (**Table 3.3**) (**Figure 3.4 (a)** and **(b)**). More specifically, the probability of chasing any individual that crossed the damselfish territory decreases (0.927 = exp (-0.076)) with the increase in intruder's size ($z = -2.662$, $p = 0.008$). Also, the probability of chase decreases (0.148 = exp (-1.909)) when performed by *S. pictus* individuals ($z = 4.613$, $p = 0.001$).

Table 3.3 - Summary table for *glm* using binomial distribution relatedly to chases. Significant values ($p < 0.05$) are highlighted in bold and marked with "*".

	Estimate	Standard Error	z - value	Pr(>|z|)
(Intercept)	1.784	0.589	3.024	**0.003***
Intruder's size (cm)	-0.076	0.029	-2.662	**0.008***
Damselfish species	-1.909	0.414	-4.613	**0.001***
Trophic group (others)	-0.338	0.399	-0.848	0.396

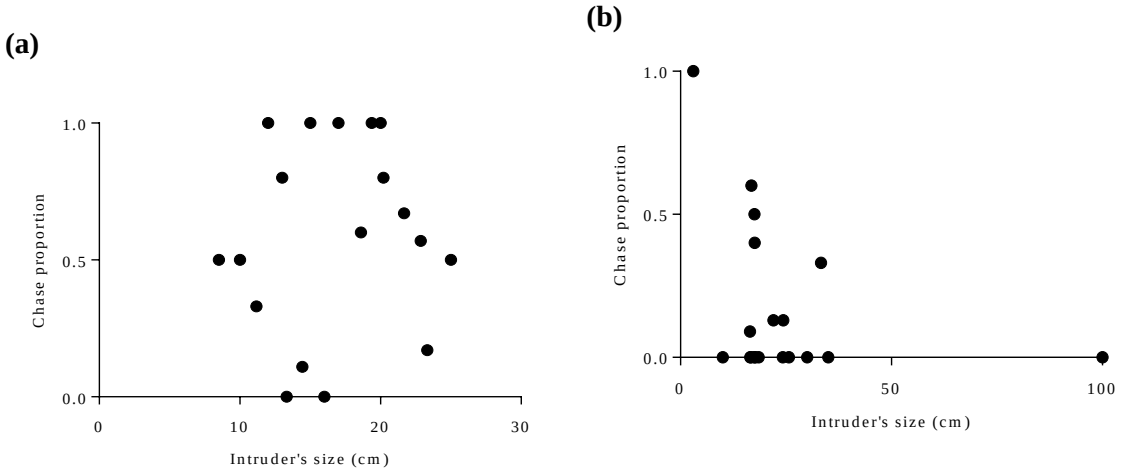

Figure 3.4 **-** Chases proportion according to intruder's size in **(a)** *S. fuscus* and **(b)** *S. pictus*. Sample size: n *S.fuscus* = 78; n *S. pictus* = 73

3.4. Cleaning behaviour

Of all 40 observed damselfish, 8 had a cleaning station inside the territory: 6 of them inside *S. fuscus* territories and 2 inside *S. pictus* territories. Cleaning gobies density per cleaning station varied between 0-2 for *S. fuscus* and between 0-3 for *S. pictus*. Cleaning events were only observed twice and only in the territories of *S. fuscus*. The two interactions lasted 6 and 13 seconds, respectively.

3.4.1. Influence of cleaning stations in frequency of intrusions within damselfish territory

The presence of cleaning stations (CS) within territories had a positive significant effect on the frequency of intrusions (**Table 3.4**) (**Figure 3.5 (a)** and **(b)**). Damselfish territories that had cleaning stations had statistically significantly more intrusions (0.393) than territories without a cleaning stations (z = 2.070, $p = 0.038$).

Table 3.4 - Summary table for *glm* using Poisson distribution relatedly to intrusions frequency. Significant values ($p < 0.05$) are highlighted in bold and marked with "*".

	Estimate	Standard Error	z - value	Pr(>|z|)
(Intercept)	1.226	0.135	9.065	**0.001***
Damselfish species	0.057	0.170	0.335	0.737
Cleaning Station	0.393	0.190	2.070	**0.038***

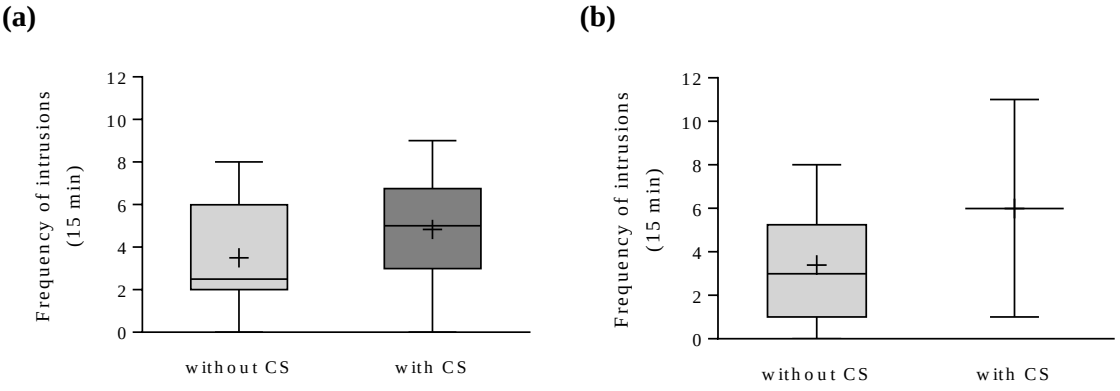

Figure 3.4 - Frequency of intrusions in damselfish territories, **(a)** *S. fuscus* and **(b)** *S. pictus*, with and without a cleaning station. *S. fuscus* territories with cleaning station within the territory. Sample size: **(a)** $n_{\text{with CS}} = 6$; $n_{\text{without CS}} = 14$ **(b)** $n_{\text{with CS}} = 2$; $n_{\text{without CS}} = 18$.

3.4.2. Ectoparasite loads (gnathiids)

Although this study focused mainly in caligids copepods and gnathiid isopods, caligids were found in residual numbers when assessing ectoparasite loads (n = 2 in 40 damselfish), therefore only gnathiid isopods were considered in the posterior analysis.

S. fuscus individuals had on average higher gnathiid loads (ectoparasites/individual) (mean = 3; min-max = 0-12) than *S. pictus* (mean = 1; min-max = 0-3) (**Figure 3.6**). Indeed, 30% of *S. pictus* were found without ectoparasites against only 15% of *S. fuscus*. However, no significant differences were found between damselfish species (Mann-Whitney, $U_{38} = 132.00$, $p = 0.068$) with respect to gnathiid loads.

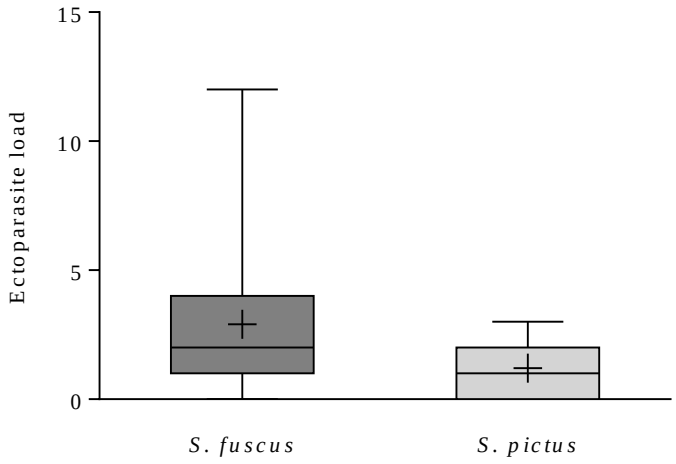

Figure 3.5 - Ectoparasite load in *S.fuscus* and *S. pictus.* Box-plots represent median, minimum and maximum and 1st and 3rd quartiles. Mean is marked as "+". Sample size for both species is 20 individuals.

3.5. Stress levels

Considering the two species studied, *S. fuscus* individuals had, on average, lower cortisol levels 1.28 ng/g (min-max = 0.10-4.50 ng/g) than *S. pictus* 1.39 ng/g (min-max = 0.05-3.00 ng/g). However, no significant differences were found between species (Mann-Whitney, $U_{33} = 208.00$, $p = 0.072$).

None of the studied variables could explain significantly the variation in damselfish cortisol levels (**Table 3.5**).

Table 3.5 - Summary table for *glm* using a Gaussian distribution relative to cortisol levels. Significant values ($p < 0.05$) are highlighted in bold.

	Estimate	**Standard Error**	**t - value**	**Pr(>|t|)**
(Intercept)	-5.403	3.702	-1.460	0.157
Gnathiids	0.581	1.159	0.501	0.621
Chases proportion	-0.747	0.678	-1.101	0.281
Intruder's size (average)	0.012	0.016	0.756	0.456
Bites	0.010	0.038	0.250	0.804
Damselfish Species	-0.086	0.738	-0.116	0.909
Temperature	0.247	0.175	1.417	0.169
Cleaning Station	-0.221	0.557	-0.397	0.695

4. DISCUSSION

4.1. Damselfish biology and territory characteristics

Damselfish is an extremely diverse fish group, with numerous species which have multiple morphological, behavioural and ecological differences among them (Robertson and Lassig, 1980). When comparing *S. fuscus* and *S. pictus* in terms of territory area there were no significant differences. Cheney and Côté (2003) verified that longfin damselfish, *Stegastes diencaeus* (Jordan and Rutter, 1897) territories, usually, have *ca.* 1 m^2, so intra-specific and interspecific differences between territory sizes were not expected. Although there were no differences in territories size between the studied damselfish species, there was, however, a great heterogeneity among individuals of the same species, which was also verified in some other studies (Menegatti *et al.*, 2003; Osório *et al.*, 2006; Barneche *et al.*, 2009). These differences may be due to several variables, such as individual body size, along with food and space availability, competitors and sexual selection (Mark A., 1980). Larger individuals may need, or are more capable to defend, larger territories (Robertson, 1996), and as there were intraspecific differences in body size this can influence the size of their territories. As these intraspecific differences were not tested in the present study, it may be important to understand, in a future study, if there is any correlation between individual body size and territory area.

Additionally, it is known that damselfish compete against each other for territories and other resources (Menegatti *et al.*, 2003; Aued, 2012; Vullioud *et al.*, 2013), as larger territories have more space for growing algae patches, that are extremely important in the feeding ecology of these species (Hixon and Brostoff, 1983; Ferreira *et al.*, 1998a). Robertson (1996) showed that interspecific competition may affect individual's territory size between different damselfish species along the Caribbean. Therefore, the territory size could also have be influenced by space availability and competitor's density, rather than only size of the individual. Individuals who arrive first choose their territory first and may expand their territory if any extra space is available in order to benefit from it, however that only occurs if they are able to defend it from other predators (Robertson, 1996).

Moreover, these differences may be related to the method used to measure the area of the territory. Although some studies have already used similar methods (Ferreira *et al.*, 1998a; Aued, 2012), this methodology is extremely dependent on the individual movements, which can be affected by other variables, like visibility conditions or water temperature. Individuals tend to be less active with low visibility conditions or lower temperatures (personal observation), so, some territory areas measured during these conditions may have been underestimated (Wenger *et al.*, 2012). On the other hand these results may also have been overrated since damselfish may occasionally explore some areas outside their territory (Robertson and Lassig, 1980).

4.2. Foraging behaviour

Damselfish are well known for their effects on benthic organisms, modifying and influencing communities of algae, mesoinvertebrates and corals, as well as the social structure of other herbivorous fish (Ferreira *et al.*, 1998a).

The feeding frequency model showed that the size of the damselfish individuals has a positive effect on the number of bites on the substratum, meaning that larger individuals take more bites on the substratum, which expected since larger individuals would need more food and, therefore, may need to have longer periods of foraging (Barneche *et al.*, 2009). Indeed, the frequency of bites on the substratum was higher for *S. fuscus* individuals than for *S. pictus*,

which can be related with all studied *S. fuscus* individuals were bigger and more robust than all the *S. pictus*. So as a result, damselfish species was not a significant variable in the model, since the variable size of individual already differentiates damselfish species.

4.3. Agonistic behaviour - intrusions

Intrusions in damselfishes' territory could affect their stress levels. For this reason it was important to explore whether both damselfish species were exposed to similar territory intrusions frequency (Creel *et al.*, 2013). Although *S. fuscus* and *S. pictus* live in slightly different depths, there is an overlap in the depth distribution at which they inhabit, so they may cohabitate. This could cause a variation on the number of individuals that enter their territories. The absence of significant differences between both species when it comes to the frequency of territory intrusions was actually expected and easily explained. The fact that different species intrude in *S. fuscus* and *S. pictus* territories does not mean there will be a difference in the frequency of these intrusions, *i.e*. species which live closer to the surface may simply intrude more often in *S. fuscus* territories, and species which live closer to the seabed may intrude more often *S. pictus* territories.

Unlike the frequency of intrusions, the size of the intruders was found to be a significant factor: the intruders in *S. pictus* territories were larger. One possible explanation for this is that, because *S. pictus* tend to be smaller than *S. fuscus*, the former do not confront larger fish as often, in order to prevent bigger disadvantages and thus tolerate them in their territories. Conversely, because *S. fuscus* are larger, they tend to chase away bigger fish letting only smaller ones in.

Setting aside the question about possible differences in frequency of intrusions, it was also important to investigate whether the intruders in *S. fuscus* and *S. pictus* territories differed in terms of trophic groups. Because *S. fuscus* and *S. pictus* are considered herbivorous, it was expected that the intruders were also mostly herbivorous fish trying to feed on their algae patches, or even trying to occupy their territory (Richard, 1971). In the case of *S. fuscus* territories, most of the intruders were mobile invertebrate feeders, and the ones which least entered their territory were planktivores. These results, although not expected, may be explained by damselfish's habit of cleaning their territory by selectively eating some algae from their algae patches, which increases the richness of benthic invertebrates and consequently, attracts more invertebrate feeders (Ferreira *et al.*, 1998a). The planktivores feed on the water column, which makes damselfish's territories less attractive since they are closer to the benthos. On the other hand, when comparing the two damselfish species, it would be more likely for planktivore fish to enter in *S. fuscus* territories instead of those of *S. pictus*, since *S. pictus* live at greater depths than *S. fuscus* and planktivores live near the surface in the water column (Helfman *et al.*, 2009). In *S. pictus* territories the most common intruders were roving herbivores and the ones which least intruded were carnivores. This result is in agreement with our hypothesis, *i.e. S. pictus* territories attract more herbivores and less carnivores, although the lack of carnivore intruders could be due to the fact that they are relatively rare in this area (Ferreira *et al.*, 2004; Floeter *et al.*, 2006).

4.4. Agonistic behaviour - chases

Territorial behaviour is a conspicuous determinant of social organization in many reef fishes including damselfish. Chases are a behavioural response directly linked with aggression (Richard, 1971). According to behavioural observations it was expected that *S. pictus* would

make fewer chases than *S. fuscus*, and the model confirms that the probability of chasing an intruder decreased when performed by *S. pictus*. These results confirm behavioural observations that *S. fuscus* was much more reactive than *S. pictus* in Arraial do Cabo during the time the study took place.

Nevertheless chase frequency can be influenced by several factors, such as intruder's size. It is known that confrontations will only occur if the competitive situation are "economically defendable" (Brown, 1964), so it was expected that larger intruders would be chased fewer times since it would be more difficult for the territory holder to defend it from a large intruder. Indeed, the probability of chase decreased with the increase in intruder size, which confirms our hypothesis.

Furthermore, it was expected that damselfish species would preferentially chase certain trophic groups more than others, such as herbivorous fish that may enter their territories in search for food. On the other hand, trophic groups such as carnivorous fish, were expected to be less chased as they may threaten the survival of territorial holders.

Although there might be differences in chase frequency according to intruder's trophic group, this was not verified even though there were differences in trophic groups intruding in damselfish territories, so it seems that damselfish chase frequency depends more on intruder's size rather than intruder's trophic group.

4.5. Cleaning behaviour

Cleaning stations play an important role in marine ecosystems reducing ectoparasite loads of individuals and also possibly reducing their stress levels (Bshary *et al.*, 2007; Soares *et al.*, 2011). Considering this, it was expected that the presence of cleaning stations within damselfish territories would influence the frequency of intrusions in a given territory, since there would be a greater demand by other species for cleaning, in order to lower their ectoparasite loads (Arnal and Côté, 1998). Indeed, territories that include a cleaning station suffered more intrusions than territories without a cleaning station. However, the sample size is quite small and not homogeneous, so no generalizations can be made with these results, as only eight of the studied territories had cleaning stations (two within *S pictus* and six within *S. fuscus* territories, the former with only two cleaning events with a duration of 6 and 13 seconds, respectively).

Contrary to what was expected cleaning events were very scarce during behavioural observations. This can be explained by the fact that the observations may have been made in a period in which cleaning gobies were not so active. Sikkel and colleagues (2004), found that longfin damselfish (*Stegastes diencaeus*) spent more time posing and being inspected by cleaning gobies at dawn (05h30-07h00). This could explain the lower number of cleaning events observed, so in the future studies involving behavioural observations should take this aspect into account.

Different species can have different parasitic loads depending on factors such as proximity to the bottom (gnathiids, for example, have a benthic larval phase) (Tanaka, 2007). Thus, one would expect that territorial species which live closer to the bottom, as is the case of damselfish, would have higher parasitic loads than planktivorous species, which spend most of their time feeding in the water column. In Arraial do Cabo, *S. pictus* tends to live slightly deeper than *S. fuscus*, and this could have an influence in ectoparasite loads, promoting higher loads in *S. pictus*. However, both species had similar ectoparasitic loads, probably because they have similar behaviour and the difference in depth was not sufficient to promote such differences.

Both species live close to the bottom regardless of the depth at which they live, so maybe, depth it not an important variable such as proximity to the bottom. Besides, smaller species tend to have less ectoparasite loads because they also have a smaller body surface area (Cheney and Côté, 2005). Although *S. fuscus* individuals were bigger than *S. pictus*, this difference did not seem to be enough to lead to significant differences in ectoparasite loads.

4.6. Stress levels

Stress levels can be influenced by several variables, internal or external to the individuals, such as temperature, ectoparasitic load, frequency of chases and intruder size, number of bites in the substrate, and damselfish species. Each of these variables may influence differently stress levels, often measured by cortisol levels (Nusbaumer, 2013).

Linking stress directly to ectoparasites infestation has been reported in numerous studies in aquaculture environments, such as salmon farms, known to have extremely high levels of caligid copepods leading to significant increases of cortisol levels (Grimnes and Jokobsen, 1996; Bowers *et al.*, 2000; Fast *et al.*, 2006; Sivertsga *et al.*, 2007). However, the link between ectoparasite infestation and stress increase in the wild (namely gnathiids) has been more difficult to demonstrate. For instance, Grutter and Pankhurst (2000) did not find an effect of parasitism on *Hemigymnus melapterus* plasma levels of cortisol or glucose. However, in laboratorial conditions, Triki and colleagues (2016) found that gnathiid-exposed fish had higher blood cortisol levels when compared with controls.

Still, it was expected that ectoparasite loads would have an influence on cortisol levels, however this was not detected in the model. Perhaps, this variable alone was not enough to influence stress levels, since the individual's ectoparasite loads observed were low (two or three gnathiids per damselfish) or maybe because gnathiids are not as harmful as other ectoparasite species, which once again confirms the difficulty in establishing a direct connection between gnathiid loads and host stress levels as shown in other previous studies (Grutter and Pankhurst, 2000). Additionally, whole-body cortisol tests performed, refer to muscular cortisol which provides information not so much of the individual's response to the catch, or even what happened on the day, but perhaps more to the habitat, diet and even growth (and sex-reproduction) (Barcellos *et al.*, 2007). As a result, no relationship between ectoparasite loads and cortisol levels was found in the present study.

Notwithstanding, other variables may contribute to host cortisol levels dynamics, namely diet, growth levels, reproduction investment and habitat conditions (depth ranges or territorial quality). Within the territorial quality, the nearby location of cleaning stations, breeding partners and the levels of constant intrusions (from conspecifics and alospecifics) could be good candidate variables, contributing to damselfish cortisol and health levels.

The frequency of intrusions in damselfish territories induces several responses at the individual level, influencing their behaviour as well as stress levels (Creel *et al.*, 2013). It would be expected that individuals which suffer more intrusions in their territory would be more stressed than individuals which suffer less intrusions. Although there were no significant differences in intrusions frequency between damselfish species in study, this variable can be affected by other variables such as the presence or absence of a cleaning station within the territory. Additionally it was expected that individuals which were visually more reactive (performed more chases to their intruders) regardless of the frequency of intrusions in their territories may have higher cortisol levels than individuals which make fewer chases. Intrusions may have an influence in stress levels since territory holders feel that their resources may be threatened. Therefore, considering the behavioural observations, knowing that the frequency of chases was different

among damselfish species studied, it was expected that *S. fuscus* being more reactive may have higher cortisol levels in comparison with *S. pictus*.

On the other hand, it is important to realize that the absence of a behavioural response to an intrusion (chase) does not always mean an absence of physiological response and low levels of stress. Territorial species need to constantly balance the costs and benefits of their behaviour (Clifton, 1990; Santangelo *et al.*, 1997; Vullioud *et al.*, 2013). Small territorial species will only chase intruders up to a certain size so that an eventual confrontation is not detrimental to their own and may expel the intruder. Sometimes, if intruders are larger than the territorial fish, the latter may exhibit a freezing behaviour simply ignoring the intruder. Yet, this reaction remains a response to an external stimulus (intrusion) and may also be associated with an increase in cortisol levels. Nevertheless the relationship between chases and an increase in cortisol levels has not still been well established (Grutter and Pankhurst, 2000a).

Habitat conditions and abiotic factors such as the presence of cleaning stations within the territories and temperature conditions may also have an important role in cortisol variations. The presence of cleaning stations within damselfish territories had a positive effect on the frequency of intrusions, promoting the intrusion of individuals seeking to be cleaned, and therefore it would be expected that this variable could also have an influence on cortisol levels, since more intrusions could mean an increase of stress in fish (Cheney and Côté, 2001; Creel *et al.*, 2013). On the other hand the damselfish in territories with cleaning stations benefit from having an easier access to cleaning gobies and may reduce their ectoparasite loads and therefore their cortisol levels may be lower than damselfish without a cleaning station within their territory. Cheney and Côté (2003b) found that apparently longfin damselfish living more than two meters from a cleaning station never visit it. Travelling even short distances from the territory appears to be costly to these species because of territorial intrusions and attacks by other conspecifics and territory holders.

Fishes are very sensitive to water temperature variations and can only tolerate a range of temperatures (Johnston and Dunn, 1987; Hanna *et al.*, 2008). These variations have an influence on individuals' homeostasis and can induce physiological and behavioural responses, affecting fish populations through changes in the balance between mortality, growth and reproduction rates (Chrousos and Gold, 1992; Barcellos *et al.*, 2007). Arraial do Cabo is known for being affected by upwelling currents often existing differences in temperature of around 3ºC. For this reason it would also be an important variable to consider in the model. However, although the temperature varied throughout the period of behavioral observations, this variable did not present an influence on cortisol levels. Possibly these changes may have been more gradual and not abrupt as in an upwelling phenomenon. Additionally, behavioural observations were performed in a short period of time and were not very dispersed so that the differences in temperature may not have been sufficient to induce a change in the individuals.

Finally, it is known that stressed individuals may have lower food intake due to the constant mobilization of energy for their vital activities, and there is some evidence that cortisol may reduce the absorption of food in the intestine suppressing growth (Bernier *et al.*, 2004). As a result a negative effect of the bites would be expect in the model.

Differences in visual behaviour that initially suggested possible differences in stress were not enough to deduce this. These behavioural differences, while visually easy to detect, are very subjective in inferring differences in stress levels of individuals.

None of the studied variables seem to have an influence on individuals' cortisol levels. Regarding chase frequency, individuals from different species may be more or less permissive in terms of intrusions in the territory, as observed in *S. pictus* (for example, damselfish of the

Caribbean are more aggressive and react more to intrusions (Wachter, 2009)). It is known that intruder's size has an influence in chase frequency by territory holders, but perhaps this variable is not sufficient to promote an increase in cortisol levels. The variable "bites on the substratum" also does not presented any type of relation with cortisol levels, which could be explained since food is a vital process of individuals and possibly will only be affected if the individuals are experiencing chronic and prolonged stress.

The presence of cleaning stations can influence the frequency of intrusions in the territories but may not be enough to increase cortisol levels. Additionally, damselfish cortisol levels, possibly increased by the entry of intruders in the territory, may be counterbalanced by the decrease in ectoparasite loads they may have, due to easier access to cleaners (Cheney and Côté, 2005). Unfortunately, this data were not possible to verify because of the reduced and heterogeneous sampling that occurred in this study.

In the future, to overcome the difficulties imposed to this study, it would be important to continue similar studies, attempting to get a larger sample with equal number of territories with and without cleaning stations, in order to evaluate the real effect of cleaning stations in damselfish territorial behaviour and its consequences on ectoparasite loads. Furthermore, behaviour observations should be performed earlier in the day to coincide with the peaks of cleaning activity and gnathiid emergency from the substratum. It would also be important to test a different methodology for cortisol analyses, such as blood samples from each damselfish observed, to better understand which variables can influence these levels. Plus, blood samples would give more accurate results for cortisol levels, providing information about each individual's response to stimuli occurring during behavioural observations and capture. In addition it would be interesting to compare the results of this study with those of previous studies regarding cleaning behaviour, ectoparasite load and damselfish behaviour.

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